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Early and Strong Immune Responses Are Associated with Control of Viral Replication and Recovery in Lassa Virus-Infected Cynomolgus Monkeys▽

Sylvain Baize,[1]* Philippe Marianneau,[1] Philippe Loth,[1]† Stéphanie Reynard,[1] Alexandra Journeaux,[1] Michèle Chevallier,[2] Noël Tordo,[1] Vincent Deubel,[1]‡ and Hugues Contamin[1]†

Unité de Biologie des Infections Virales Emergentes, Institut Pasteur-IFR 128 BioSciences Gerland—Lyon Sud, Lyon, France,[1] and Biomnis, Lyon, France[2]

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Lassa virus causes a hemorrhagic fever endemic in West Africa. The pathogenesis and the immune responses associated with the disease are poorly understood, and no vaccine is available. We followed virological, pathological, and immunological markers associated with fatal and nonfatal Lassa virus infection of cynomolgus monkeys. The clinical picture was characterized by fever, weight loss, depression, and acute respiratory syndrome. Transient thrombocytopenia and lymphopenia, lymphadenopathy, splenomegaly, infiltration of mononuclear cells, and alterations of the liver, lungs, and endothelia were observed. Survivors exhibited fewer lesions and a lower viral load than nonsurvivors. Although all animals developed strong humoral responses, antibodies appeared more rapidly in survivors and were directed against GP_1, GP_2, and NP. Type I interferons were detected early after infection in survivors but only during the terminal stages in fatalities. The mRNAs for CXCL10 (IP-10) and CXCL11 (I-TAC) were abundant in peripheral blood mononuclear cells and lymph nodes from infected animals, but plasma interleukin-6 was detected only in fatalities. In survivors, high activated-monocyte counts were followed by a rise in the total number of circulating monocytes. Activated T lymphocytes circulated in survivors, whereas T-cell activation was low and delayed in fatalities. In vitro stimulation with inactivated Lassa virus induced activation of T lymphocytes from all infected monkeys, but only lymphocytes from survivors proliferated. Thus, early and strong immune responses and control of viral replication were associated with recovery, whereas fatal infection was characterized by major alterations of the blood formula and, in organs, weak immune responses and uncontrolled viral replication.

Lassa fever is a severe hemorrhagic fever endemic in West Africa: there are 300,000 cases annually, leading to 5,000 to 6,000 deaths (46). There is also sporadic importation of cases into industrial countries. The etiologic agent is Lassa virus (LV), an old-world *Arenavirus* belonging to the family *Arenaviridae* (6). It is an enveloped virus composed of two negative-strand RNA segments. The large segment codes for a small zinc-binding (Z) protein involved in the regulation of transcription and replication and in the budding of viruses (11, 59) and for RNA polymerase (L); the small segment encodes the nucleoprotein (NP) and the two envelope glycoproteins (GP_1 and GP_2), allowing cell entry by α-dystroglycan binding and consecutive fusion (8, 57). Although several candidates have been described (9, 16, 21, 37), there is no licensed vaccine against LV, and the only effective antiviral drug, ribavirin, has to be administered very early after infection, limiting its value in countries where the virus is endemic (44). Humans are infected through contact with a peridomestic rodent, the mouse *Mastomys natalensis*, which is the reservoir host for LV (46). Human-to-human transmission then occurs via mucosal/cutaneous contact or nosocomial contamination (19). The severity of the disease is variable, from asymptomatic infections, which are frequent, to fatal hemorrhagic fever. Nonspecific signs, including fever, headache, arthralgia, myalgia, and severe asthenia, appear 6 to 12 days after infection. A few days later, abdominal pain, pharyngia, cough, conjunctivitis, diarrhea, and vomiting are observed. The most severe cases may involve cervical and facial edema, hemorrhage, renal and liver failure, and encephalopathy. Death occurs in a context of global failure with hypotension, hypovolemia, and hypoxic shock (15). In survivors, symptoms disappear 10 to 15 days after onset, but transitory or permanent deafness affects about one-third of cases (13).

The pathogenesis of Lassa fever remains unclear. Dendritic cells, macrophages, hepatocytes, and endothelial cells are the main targets for LV replication (2, 36, 41, 68). However, the changes described in the endothelium and in other organs are not severe enough to account for terminal shock and death, which seem instead to depend on the host response. The most frequent microscopic alterations are multifocal hepatocellular necrosis with minimal recruitment of inflammatory cells, interstitial pneumonitis, acute myocarditis, and damage to reticuloendothelial tissues (15). Little is known about the immune responses induced during Lassa fever in humans. Infection seems to be controlled primarily by T cells (60, 61). Recovery does not correlate with production of specific immunoglobulin G (IgG) (31), and neutralizing antibodies (NAbs) are detected only after recovery, and then at low titers (25). In contrast,

* Corresponding author. Mailing address: UBIVE, Institut Pasteur, 21 av. Tony Garnier, 69365 Lyon cedex 7, France. Phone: (33) 437 28 24 44. Fax: (33) 437 28 24 41. E-mail: sylvain.baize@inserm.fr.

† Present address: MDS Pharma Services, Les Oncins, 69210 Saint Germain sur l'Arbresle, France.

‡ Present address: Institut Pasteur of Shanghai, Chinese Academy of Sciences, 225 South Chongqing Road, Shanghai 200025, China.

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severe LV infections are associated with elevated viremia and immunosuppression. Cellular depletion of lymphoid tissues, necrosis of the splenic marginal zone, and transitory lymphopenia have been described in patients (15, 17). Dendritic cells massively release LV particles but are not activated and do not produce cytokines, probably leading to defective T-cell responses (2, 41). Similarly, macrophages are productively infected with LV without activation, with the exception of modest type I interferon (IFN) production (2, 3). Thus, LV tropism for antigen-presenting cells probably contributes to the defective immune responses observed in severe cases.

The locations of zones of endemicity and the high infectivity of LV have hampered investigations of Lassa fever in humans. There is no rodent model, except for strain 13 guinea pigs, which can be infected but unfortunately do not reproduce the physiopathology or immune responses observed in humans (28). Therefore, nonhuman primates are still the only relevant model for Lassa fever: rhesus and cynomolgus monkeys (*Macaca mulatta* and *Macaca fascicularis*, respectively) are the primates that have been most extensively used (7, 16, 21, 26, 27, 29, 58, 65). These closely related nonhuman primates both reproduce the systemic disease involving most of the visceral organs, with microscopic lesions, including hepatocellular necrosis, interstitial pneumonia, arteritis, and encephalitis. In both humans and macaques, the severity of LV infection is correlated with viremia, increased blood transaminase levels, and hemorrhagic signs. The hematologic and hemostatic disorders are also similar, and death occurs in both species in the context of hypovolemic and hypotensive shock and acute respiratory distress (18). Marmosets have recently been reported as an alternative nonhuman primate model for LV infection (10), and lymphocytic choriomeningitis virus (LCMV) has been used as a surrogate for LV in the macaque model (35, 39). Here, we report the infection of cynomolgus monkeys with a primary strain of LV, the AV strain (23), and describe the virological and immunological parameters associated with fatal and nonfatal outcomes.

MATERIALS AND METHODS

Virus. LV (strain AV, a generous gift from S. Becker) (23) from the serum of a patient with fatal outcome was passaged four times in Vero E6 cells at 37°C. The cell supernatant was used as LV stock (10^7 focus-forming units [FFU]/ml). The cell supernatant of uninfected Vero E6 cells was used as a mock control with uninfected monkeys. Tests were performed to verify that cell lines and viruses were free of mycoplasmas. All experiments with infectious LV were conducted in biosafety level 4 facilities (Laboratoire P4 Jean Mérieux, Lyon, France).

Animals and virus inoculations. Eight healthy cynomolgus monkeys were purchased from a primate quarantine center (Bioprim, Toulouse, France) and housed in biosafety level 4 facilities for 1 week before experiments were started. The animals were 4-year-old males with normal weights and activity levels. All the procedures for animal handling were performed in accordance with the regulations of the ethics and animal use committee of the Région Rhône-Alpes and of the European Community. Six monkeys were subcutaneously injected with LV, three of them with a low dose (10^3 FFU) and the other three with a high dose (10^7 FFU) of LV (all injections administered were 0.5 ml). Two monkeys were mock infected and used as negative controls.

Biological samples and clinical, hematological, and biological analyses. The monkeys were monitored twice daily for clinical signs. Animals that were moribund were euthanized by intravenous injection of pentobarbital (Dolethal; Vétoquinol, Oxfordshire, United Kingdom), and a complete necropsy with tissue harvest was performed. Survivors and controls were killed about 1 month after infection. Until euthanasia, the animals were sedated at about 3-day intervals with tiletamin/zolazepam (Zoletil; Virbac, Carros, France) to record body temperature and collect blood samples. Total white blood cell and platelet counts, hematocrit, and total hemoglobin values were determined with EDTA-containing blood using a hematological analyzer (VetTest; Idexx, Eragny sur Oise, France). Concentrations of electrolytes (Na^+, Cl^-, and K^+), albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, γ-glutamyl transferase, glucose, total protein, total bilirubin, urea nitrogen, and creatine in serum samples were determined using a biochemical analyzer (VetTest; Idexx).

Virus titration. LV particles in serum and the supernatants of crushed tissues from infected monkeys were titrated as previously described (3). The limit of detection was 200 FFU/ml of plasma and 40 FFU/mg of tissue.

Quantification of viral RNA in plasma and organs by quantitative RT-PCR. Viral RNA was extracted from plasma with the Qiamp viral-RNA isolation kit (Qiagen, Courtaboeuf, France). Pieces of tissues and organs were weighed and shaken for 3 min at 1,500 RPM with 0.75- to 1-mm glass beads in a Retsch MM200 crusher (Fisher-Bioblock Scientific, Illkirch, France). Viral RNA was extracted from crushed samples with the RNeasy RNA isolation kit (Qiagen). Viral NP RNA was reverse transcribed, and the resulting cDNA was amplified and quantified using TaqMan one-step reverse transcription (RT)-PCR master mix reagents (Applied Biosystems, Courtaboeuf, France) and a synthetic standard RNA as previously described (3).

Detection of mRNA by real-time RT-PCR. Peripheral blood mononuclear cells (PBMC) were isolated from EDTA-containing blood or from crushed lymph nodes by density gradient centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) and washed twice in RPMI 1640-Glutamax I supplemented with 10 mM HEPES, 1% nonessential amino acids, 1% penicillin-streptomycin, and 10% fetal calf serum (FCS) (C-RPMI) (all from Invitrogen). Cellular RNA was isolated and used for the synthesis of first-strand cDNA as previously described (2). The cDNA was amplified on an ABI Prism 7000 real-time thermocycler (Applied Biosystems), using SYBR green PCR master mix (Applied Biosystems) and the following primers: β-actin, 5′-TGAACCCCAAGGCCAACC-3′ and 5′-GCCAGCCAGGTCCAGACG-3′; CXCL10, 5′-TGAAAAAGAAGGGTGAGAAGAGGT-3′ and 5′-TGATGGCCTTAGATTCTGGATTC-3′; CXCL11, 5′-TACGGTTGTTCAAGGTTTCCC-3′ and 5′-TGGAGGCTTTCTCAATATCTGC-3′. The specificities of the amplicons were verified by determining their melting temperatures. For quantification of type I IFN mRNA, TaqMan assays were carried out with the following primers and probes: β-actin, 5′-GCGCGGCTACAGCTTCA-3′ and 5′-CTTAATGTCACGCACGATTTCC-3′, probe, 5′-CACCACGGCCGAGC-3′; beta IFN (IFN-β), 5′-TCTCCACTACGGCTCTTTCCA-3′ and 5′-ACACTGAAAACTGCTGCTTCTTTG-3′, probe, 5′-AACTTGCTTGGATTCCT-3′; IFN-α1, 5′-GTGGTGCTCAGCTGCAAGTC-3′ and 5′-TGTGGGTCTCAGGCAGATCAC-3′, probe, 5′-AGCTGCTCTCTGGGC-3′; and IFN-α2, 5′-CAGCCTAGCAGCATCTGCAATAT-3′ and 5′-CCAGGGCCACCAGTAAAGC-3′, probe, 5′-ACAATGGCCTTGACCTT-3′. The cDNA was amplified using the TaqMan Universal Master Mix (Applied Biosystems). The amounts of chemokine/cytokine mRNA relative to that of β-actin mRNA in each sample (relative mRNA levels) were calculated as follows: ΔC_T (cycle threshold) = $C_{T\text{gene X}} - C_{T\beta\text{-actin}}$; mRNA of interest/β-actin mRNA = $2^{-\Delta Ct}$.

Histology. Pieces of the various tissues harvested during necropsy were fixed in 10% formalin and embedded in paraffin. The tissue pieces were cut into 5-μm sections, the paraffin was removed, and the sections were stained with hematoxylin and eosin.

Detection of antibodies by ELISA and Western blotting. To detect LV-specific IgM and IgG by enzyme-linked immunosorbent assay (ELISA), Maxisorp plates (Nunc, Copenhagen, Denmark) were coated overnight with goat antibody against monkey IgM or IgG (Kirkegaard & Perry Laboratories [KPL], Gaithersburg, MD), respectively. Dilutions of plasma samples were incubated on the plate for 1 h at 37°C, and supernatants of LV- or mock-infected Vero E6 cells were applied; LV-specific polyclonal mouse ascitic fluid was then added, and the plates were incubated for 1 h at 37°C. Horseradish peroxidase-conjugated goat antibody against mouse IgG (Sigma-Aldrich, Saint-Quentin Fallavier, France) was added, and the plates were developed with TMB substrate (KPL). The results are expressed as optical densities.

To detect specific IgG by Western blotting, LV NP was obtained from lysates of SF9 cells infected by a recombinant baculovirus (Invitrogen), and LV GP_1 and GP_2 were obtained from retroviral pseudoparticles. LV antigens were diluted in Laemmli buffer (Bio-Rad, Marnes-la-Coquette, France), heated for 10 min at 96°C, and separated by reducing 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with molecular weight standards (Rainbow; Amersham Biosciences, Saclay, France). After electrophoresis, the proteins were transferred onto a nitrocellulose membrane by application of 160 mA for 90 min. The membranes were blocked with 2.5% milk in phosphate-buffered saline (PBS)-0.05% Tween 20 for 1 h at room temperature. Monkey plasmas diluted 1:100 or 1:500 in PBS-2.5% milk-0.1% Tween 20 were incubated with the membrane for

90 min at room temperature. LV-specific IgG was detected with horseradish peroxidase-conjugated goat anti-monkey IgG (gamma specific) (KPL) at a dilution of 1:1,500 and the substrate diaminobenzidine (Sigma-Aldrich).

NAb assay. The presence of NAbs in plasma was evaluated by incubating several dilutions of LV- or mock-infected monkey plasmas with 100 FFU of LV for 30 min at 37°C. Residual infectious LV particle titers were determined as previously described (3).

ELISA for cytokines. IFN-α in plasma was assayed with a human-specific ELISA set (Bender MedSystems, Vienna, Austria). Interleukin-1β (IL-1β) and IL-8 in plasma were detected using a monkey-specific ELISA kit (Bender MedSystems). IL-2, IFN-γ, tumor necrosis factor alpha (TNF-α), and IL-6 were quantified with a monkey-specific ELISA set (BD OptEIA; BD-Biosciences, Le Pont de Claix, France).

Flow cytometry. Aliquots of 50 μl of whole blood containing EDTA were stained for 30 min at 4°C with the following monoclonal antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (clone SP34), anti-CD14 (M5E2), and anti-CD20 (2H7); phycoerythrin (PE)-conjugated anti-CD69 (FN50), anti-CD80 (L307.4), and anti-CD25 (M-A251); peridinin chlorophyll protein-conjugated anti-CD4 (L200) and anti-CD8 (RPA-T8); PE-Cy5-conjugated anti-HLA-a, -b, and -c (G46-2.6); and CyChrome-conjugated anti-CD28 (CD28.2) and anti-CD95 (DX2) (all from BD Biosciences). Isotypic controls were performed by staining cells with FITC-conjugated mouse IgG1 (MOPC-21), PE-conjugated mouse IgG2a (G155-178), and CyChrome-conjugated mouse IgG1 (MOPC-21) (BD Biosciences). Red blood cells were then lysed, and PBMC were fixed using the Immunoprep kit and a Q-Prep apparatus (Beckman-Coulter, Villepinte, France). An EPICS-XL four-color cytometer (Beckman-Coulter) with Expo 32 ADC software (Beckman-Coulter) was used for flow cytometry. The percentages of lymphocytes, monocytes, and granulocytes were determined by gating the respective populations using forward and side scatter parameters.

In vitro analysis of LV-specific T-cell responses. Inguinal lymph nodes were surgically removed from monkeys 9 days after infection and then lacerated with a scalpel and crushed in a petri dish containing C-RPMI. This cell suspension was filtered through a 100-μm-pore-size nylon cell strainer (BD Biosciences), and the T lymphocytes were isolated by density gradient centrifugation on Ficoll-Paque (Pharmacia). The cells were resuspended in 90% FCS and 10% dimethyl sulfoxide and stored in liquid nitrogen until they were used. The cells were thawed, washed three times in C-RPMI, and counted using trypan blue staining. The cells were pelleted by centrifugation and resuspended in uninfected Vero E6 cell supernatant (mock) or in heat-inactivated LV-infected Vero E6 cell supernatant and incubated for 1 h at 37°C. They were then cultured in C-RPMI at a density of 10^6 cells/ml. The cells were harvested 3 days after stimulation, washed in PBS-2.5% FCS, incubated for 15 min in PBS-5% AB^+ human serum, and stained for 30 min at 4°C with the following monoclonal antibodies: FITC-conjugated anti-human CD25 (M-A251), peridinin chlorophyll protein-conjugated anti-human CD3 (SP34-2), and PE-Cy7-conjugated anti-human CD8 (RPA-T8) (all from BD-Biosciences). The cells were washed in PBS-2.5% FCS, incubated in Cytofix/Cytoperm buffer (BD-Biosciences) for 20 min at room temperature, and washed in Cytoperm buffer (BD Biosciences). PE-conjugated anti-human KI67 (BD Biosciences) was then added, and the samples were incubated in Cytoperm buffer for 30 min at room temperature, after which the cells were washed in Cytoperm buffer, resuspended in PBS-1% paraformaldehyde, and analyzed by flow cytometry.

Statistical analysis. Student's *t* test and the Mann-Whitney rank sum test were used to compare data sets. SigmaStat 3.5 (Systat Software, Erkrath, Germany) was used for statistical calculations.

RESULTS

Clinical observations. Three cynomolgus monkeys were inoculated subcutaneously with 10^3 FFU of the AV strain of LV (23), and three additional monkeys were similarly inoculated with 10^7 FFU of the same virus. Clinical signs were unremarkable until 6 days after infection, when weight loss and hyperthermia appeared. Behavioral changes with anorexia and depression were also observed. The animals lost nearly 10% of their body weight by 12 to 16 days after infection, and fever peaked on days 9 to 12 but was low grade (up to 39°C) (data not shown). One animal infected with 10^3 FFU of LV died (16 days after infection), and another was killed when moribund (21 days after infection); these two animals presented an altered clinical state from 10 days after infection, with severe depression, acute respiratory syndrome, neurological disturbances, and a body temperature that declined to subnormal levels prior to death. The dying animal was euthanized because he had reached the end points defined in agreement with the Ethical Committee. At the time of euthanasia, this monkey had lost 18% of its body weight, had been in hypothermia (36°C) for at least 2 days, and presented severe neurological signs (data not shown). In contrast, the third monkey infected with 10^3 FFU and all the animals infected with 10^7 FFU recovered completely, with all symptoms disappearing by about 21 days after infection. These surviving monkeys and the mock-infected monkeys were euthanized 28 to 36 days after infection, and necropsies were performed.

Biological and hematological alterations. There was a transient but large increase of AST and ALT concentrations in plasma between 9 and 22 days after infection; the increase was particularly pronounced in fatally infected animals and also in one monkey infected with a high dose of virus (Fig. 1A and B). The other biochemical markers analyzed remained in their normal ranges. All infected monkeys suffered early (from 3 days after infection) thrombocytopenia (Fig. 1C), and the intensities were similar in all infected animals. However, thrombocytopenia was compensated for in survivors, with platelet levels returning to normal values 3 weeks after infection, whereas low platelet counts persisted until death in fatally infected animals. All infected animals also presented lymphopenia affecting all the lymphocyte subpopulations, including $CD4^+$ and $CD8^+$ T cells, B cells, and NK cells (Fig. 1D to G). These alterations were more pronounced in fatally infected monkeys. The lymphocyte count, however, returned to normal levels between 9 and 16 days after infection. Over the following days, leukocytosis appeared in some animals (Fig. 1D to H). Finally, a transient and large increase in the number of circulating monocytes was observed 12 to 16 days after infection, but only in surviving monkeys (Fig. 1I).

Pathological findings and histology. The necropsy of fatally infected monkeys revealed generalized adenopathy and splenomegaly, slightly enlarged livers, and major pleural extravasation, in one case with abdominal hyperemia. In contrast, no notable abnormality was detected in the surviving monkeys during necropsy 28 to 36 days after LV infection. The microscopic lesions in fatally infected monkeys were characterized by focal hepatocellular necrosis involving 1 to 5% of the hepatic parenchyma (Fig. 2A) and by substantial mononuclear infiltrates in the sinusoids (Fig. 2B). The lungs and the pleural membrane were particularly affected, with mild to intense interstitial pneumonia, acute alveolitis and endothelitis (Fig. 2C), and acute fibrino-leukocytic pleurisy (Fig. 2D). The main renal changes consisted of multifocal cortical interstitial mononuclear cell infiltrates (Fig. 2E). Lymph nodes and spleen were hyperplasic, with an intense macrophagic reaction (Fig. 2F). Hyperplasia of the white pulp and intense congestion of the red pulp were observed (Fig. 2G and H). Acute necrosing pancreatitis was detected in only one fatally infected monkey (Fig. 2I). In summary, the main alteration observed in fatally infected monkeys was substantial infiltration by mononuclear cells, mainly perivascular, which reached most organs, particularly those described above, but also the bladder, pericardium, skin, central and peripheral nervous system, and testis.

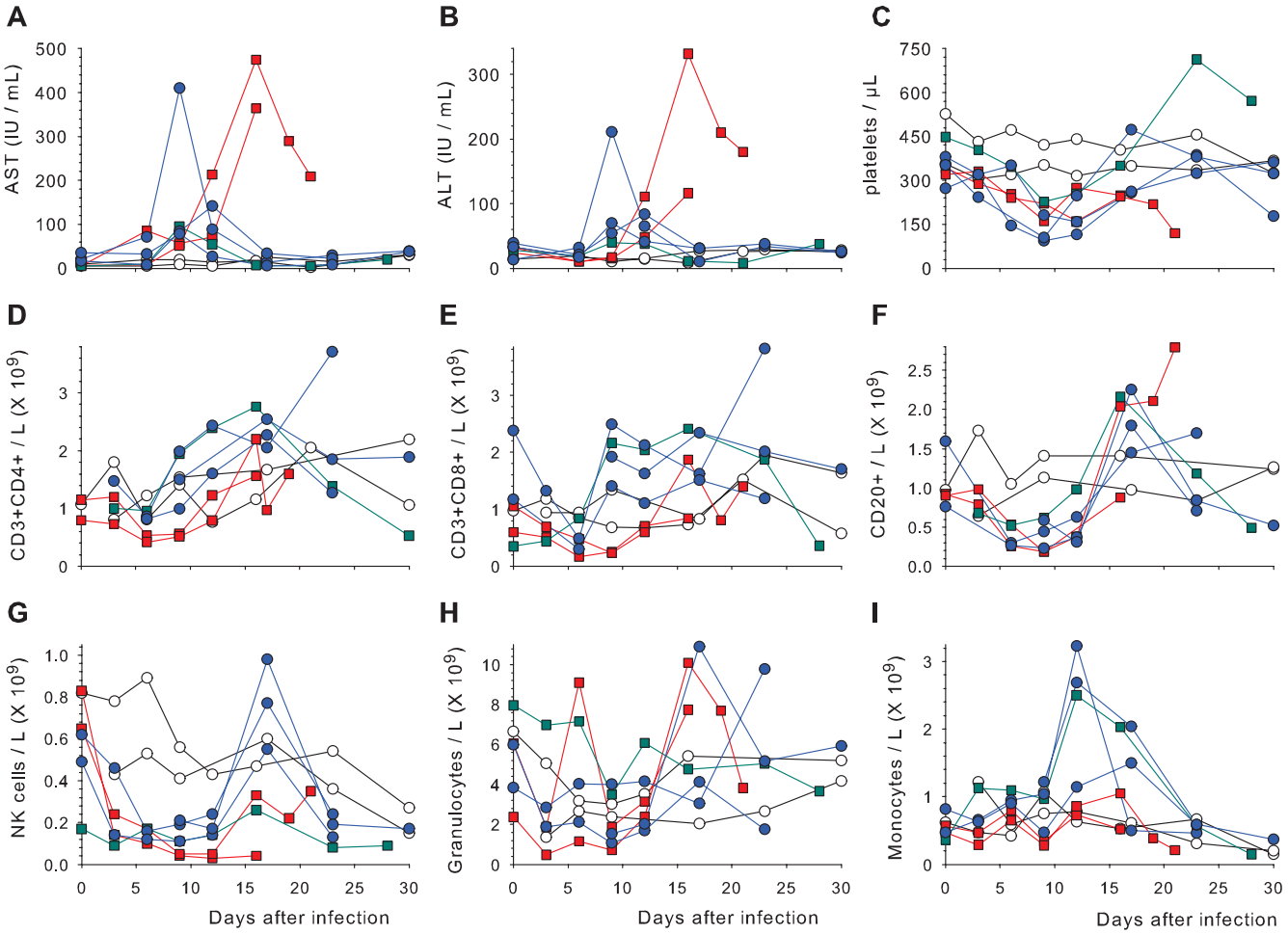

FIG. 1. Hepatic enzyme levels and blood composition during the course of Lassa fever. (A and B) Concentrations of AST (A) and ALT (B) were determined in plasma from fatally infected monkeys (red squares), the low-dose-infected survivor (green squares), high-dose-infected survivors (blue circles), and mock-infected monkeys (white circles). (C) Platelet counts were determined in the blood of monkeys during the course of the disease. (D to I) The numbers of circulating $CD4^+$ (D) and $CD8^+$ (E) T cells, B cells (F), NK cells ($CD3\text{-}CD8^+$) (G), granulocytes (H), and monocytes (I) in animals were determined by flow cytometry during the course of LV infection.

In contrast, the lesions observed in survivors were not severe, with moderate perivascular mononuclear cell infiltrates and mild hyperplasia of lymph nodes and spleen. Nevertheless, one animal infected with 10^7 FFU of LV displayed intense lymphocytic orchitis.

Viremia and viral load in tissues. We used two methods to quantify the viral load in monkeys: the quantification of viral RNA copies, which is indicative of both infectious and defective viral particle numbers, and the infectious focus numeration, which represents the number of infectious particles. LV RNA was detected as early as 3 days after infection in the circulation of nearly all monkeys. The number of RNA copies peaked between 6 and 12 days after infection in surviving monkeys and rapidly dropped thereafter, whereas the levels of viral RNA increased until death in fatally infected animals (Fig. 3A). Infectious LV particles were first detected 9 days after infection in the sera of all monkeys, and the viral load was higher in fatally infected animals than in survivors. Viremia was maximal 9 to 12 days after infection in survivors and then declined rapidly, but the viral load remained high during the days before death in fatally infected monkeys (Fig. 3B). Furthermore, the viral RNA/FFU ratio was significantly higher in survivors than in fatally infected monkeys (mean for fatalities, 205 ± 41; mean for survivors, 1,547 ± 403; $P = 0.01$; ratio of the viral stock, 190) (Fig. 3C).

A high viral load was also detected at necropsy in organs—spleen, lymph nodes, liver, kidney, lung, and brain—of fatally infected monkeys (Fig. 3D and E). Although infectious LV particles were not detected at necropsy in these organs from survivors, substantial amounts of viral RNA were found. However, it is not possible to compare these lower viral loads with those from fatalities, as the organs were obtained at different times: 17 and 21 days after infection in fatalities and after 1 month in survivors.

Humoral responses during Lassa fever. LV-specific IgM was detected in the two fatally infected monkeys from 12 days after infection, and the levels rose rapidly to reach a plateau about 16 days postinfection (Fig. 4A). Interestingly, IgM appeared as early as 9 days after infection in surviving monkeys, and the levels were maximal 3 days later. LV-specific IgG was first

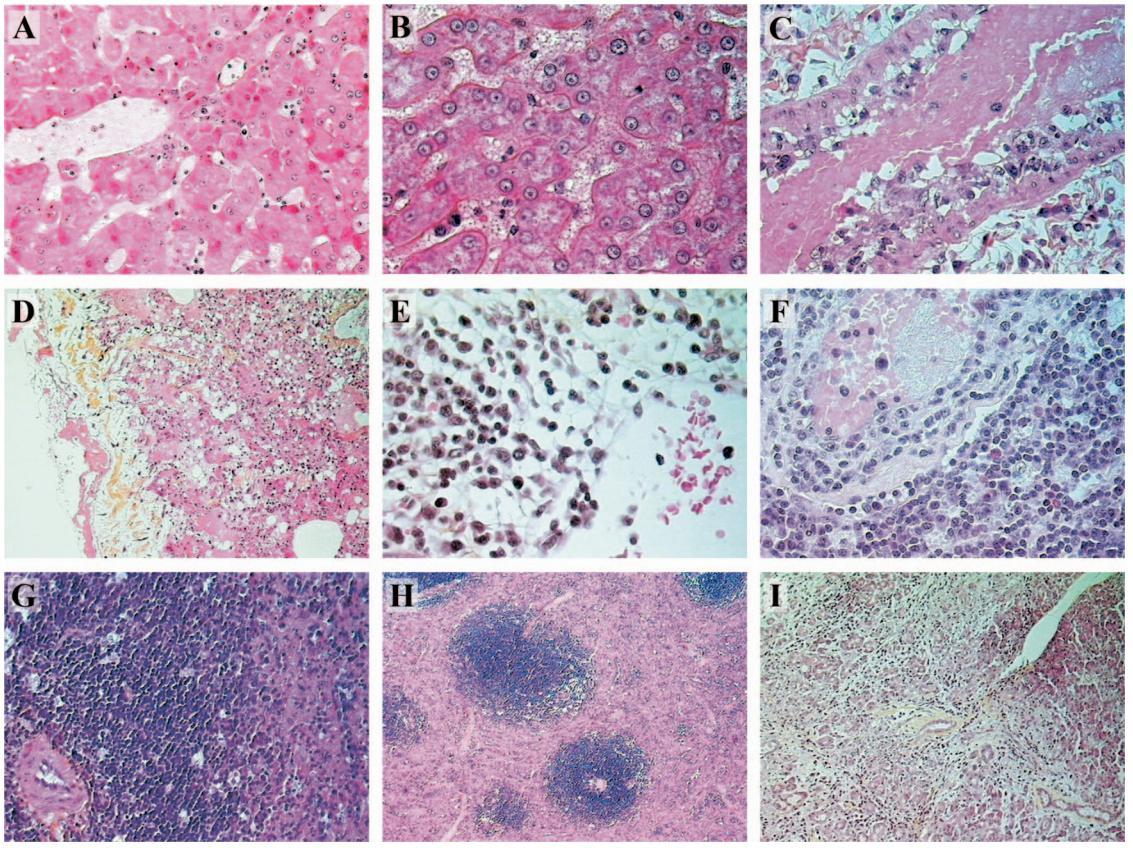

FIG. 2. Pathology in fatally infected cynomolgus monkeys. Tissues were obtained at necropsy from one monkey found dead (A to D and F to H) and from another euthanized when moribund (E and I). (A and B) Acidophilic necrosis in hepatocytes from the centrolobular zone (A) and inflammatory cell infiltrate in the sinusoids (B) of the liver. (C) Acute alveolitis and endothelitis in the lungs. (D) Fibrino-leukocytic coating on the pleural membrane. (E) Perivascular mononuclear cell infiltrate in the kidney. (F) Macrophagic hyperplasia in lymphoid follicles. (G) Hyperplasia of the splenic white pulp and numerous macrophages in the lymphoid cluster. (H) Hyperplasia of the white pulp and intense congestion of the red pulp in the spleen. (I) Substantial lesions of necrosing pancreatitis and destroyed parenchyma in the pancreas.

detected 12 days after infection in both fatally infected and surviving monkeys, but in survivors, the titers were much higher (Fig. 4B). Thus, the humoral responses seem to be induced earlier and more vigorously during nonfatal infection.

Sera were tested by Western blotting with GP and NP antigens. Both GP_1- and GP_2-specific IgG appeared between 12 and 16 days after infection (Fig. 4C). The GP-specific responses then increased rapidly in surviving monkeys, and also in the fatally infected monkey that died on day 21. NP-specific IgG was also detected from 12 to 16 days after infection in surviving monkeys, and the titers increased during the progression of the disease. However, the intensity of this response was variable, and NP-specific IgG titers were low or undetectable in fatally infected monkeys: indeed, only a very faint band was detected and only in the 1:100 dilution of plasma collected just before death from the monkey that died on day 21 (data not shown). Finally, no neutralizing antibodies were detected in LV-infected monkeys (data not shown).

Cellular immune responses in LV-infected monkeys. The expression of several molecules involved in activation at the surfaces of PBMC was analyzed by flow cytometry. The counts of $CD8^+$ T cells expressing CD69 were transiently high 3 days after infection in two of three high-dose-infected monkeys and 6 days after infection in the surviving low-dose-infected monkey, respectively, but not in fatally infected animals (Fig. 5A). CD25 was detected 9 days after infection on a large number of $CD8^+$ T cells from surviving monkeys, particularly the low-dose-infected monkey (Fig. 5A), while $CD25^+$ $CD8^+$ T cells circulated 3 days later in one fatally infected animal only. Similarly, $CD69^+$ $CD4^+$ T cells were transiently detected 6 days after infection in the blood of two of three high-dose-infected monkeys and in the low-dose-infected surviving monkey, but not in fatally infected animals. CD25 was expressed on day 9 by $CD4^+$ T cells of only two surviving monkeys infected with a low and a high dose and 12 days after infection in a moderate number of $CD4^+$ T cells from one fatally infected animal (Fig. 5B). These findings suggest that earlier and stronger T-cell responses were induced during nonfatal than during fatal infection. Finally, a large proportion of T cells from surviving monkeys and nearly all T cells from fatally infected animals did not express CD28 16 days after infection (Fig. 5C).

We then tested for the expression of CD86, CD80, HLA-DR, and HLA-a, -b, and -c at the surface of monocytes. CD86 and HLA-DR expression was not modified during the course of the disease (data not shown). In contrast, a significant transient increase of the expression of HLA-a, -b, and -c and CD95

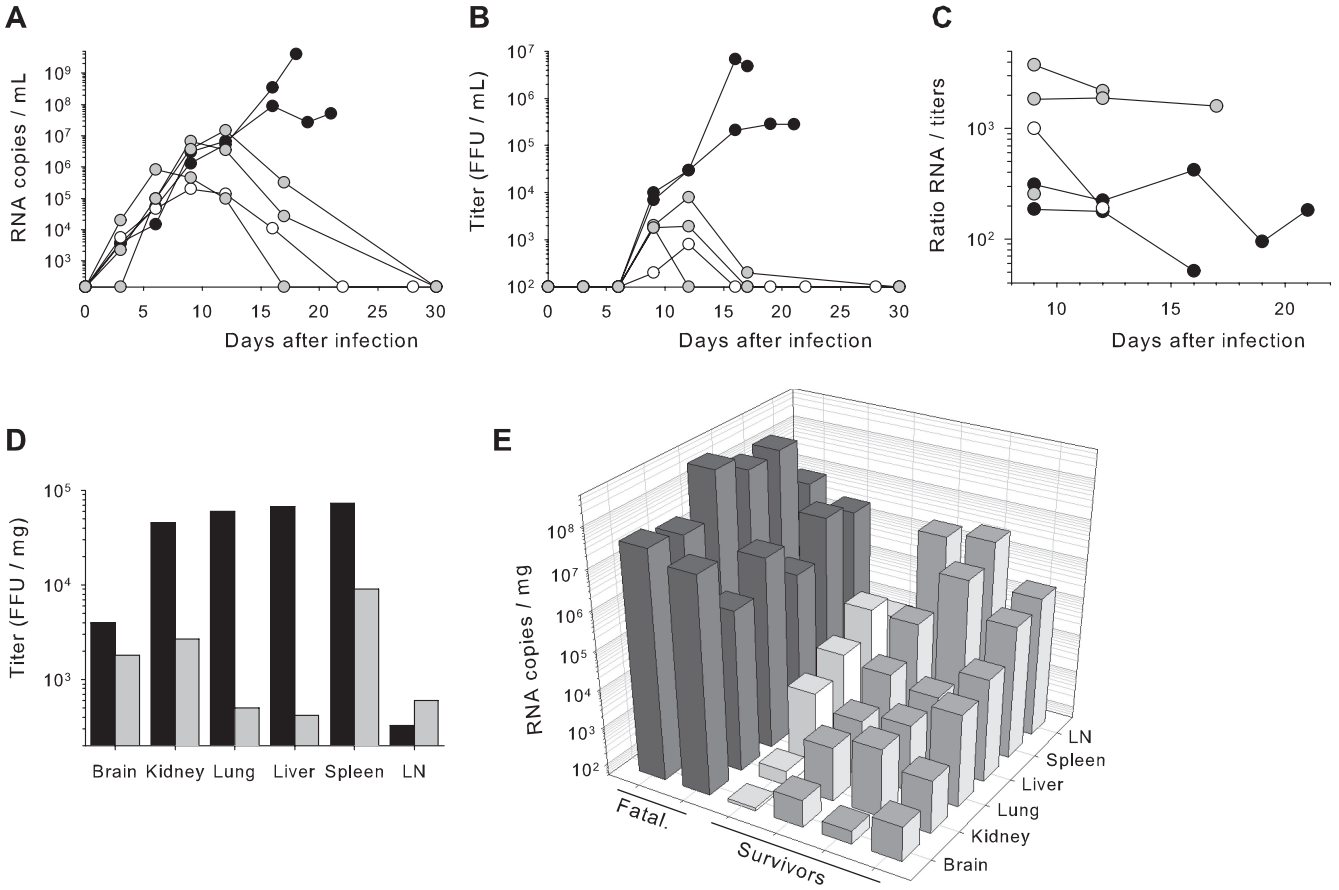

FIG. 3. Viremia and tissue viral load during Lassa fever in cynomolgus monkeys. (A) The numbers of viral-RNA copies in plasma from fatally infected monkeys (black circles), the low-dose-infected survivor (white circles), and high-dose-infected survivors (gray circles) were determined by quantitative RT-PCR. (B) The number of infectious viral particles was also determined in plasma from the same animals by titration on Vero E6 cells. (C) The ratio of viral-RNA copies to the infectious titer was calculated for plasma samples from LV-infected monkeys and is represented as in panel A. (D) Infectious viral titers in organs (LN, lymph nodes) obtained at necropsy from fatally infected monkeys were determined by titration. (E) The numbers of viral-RNA copies in organs obtained at necropsy from fatally infected monkeys (16 and 21 days after infection) (black bars), the low-dose-infected survivor (day 28) (light-gray bars), and high-dose-infected survivors (days 30 to 34) (dark-gray bars) were determined by quantitative RT-PCR.

was observed 6 days after infection in monocytes from all infected monkeys, whatever the outcome (Fig. 5D and E). In addition, a large number of $CD80^+$ monocytes circulated in the blood of surviving monkeys from 6 days after infection, returning to basal levels by days 12 to 16 (Fig. 5F). Similar changes were observed in fatally infected animals, but the numbers of monocytes involved were smaller. These observations suggest strong activation of monocytes a few days after infection, particularly in surviving monkeys.

To determine whether LV-specific T-cell responses were induced in animals, T lymphocytes derived from lymph nodes obtained 9 days after infection were stimulated in vitro with inactivated LV (LV antigen) or with noninfected Vero E6 cell supernatant (mock infection) and were analyzed 3 days later by flow cytometry. Stimulation with inactivated LV led to a greater number of KI67-expressing $CD4^+$ and $CD8^+$ T cells in samples from survivors, but not in those from fatalities or mock-infected monkeys, than in the mock-stimulated T cells (Fig. 5G). Interestingly, the percentage of proliferating ($KI67^+$) cells was higher among T lymphocytes from three of four survivors than among those from fatalities and control monkeys after mock stimulation (Fig. 5G). In addition, the proportions of CD25-positive cells among $CD4^+$ T lymphocytes from three of four survivors and one of two fatalities and among $CD8^+$ T cells from all infected monkeys were higher after stimulation with inactivated LV than among T lymphocytes from mock-infected animals (Fig. 5H). The strongest responses were observed with T cells from the survivor infected with a low dose of LV (Fig. 5G and H).

Production of cytokines and chemokines by LV-infected monkeys. We tested PBMC and lymph nodes for various mRNAs coding for cytokines and chemokines by real-time RT-PCR and assayed plasma for most of them by ELISA. No differences were found between mock- and LV-infected monkeys for either the amounts of mRNA or protein of IL-1β, TNF-α, IL-2, IFN-γ, or IL-8 or the amounts of mRNA coding for IL-4 and IL-10 (data not shown). Interestingly, although the expression of IL-6 mRNA was not induced in PBMC in lymph nodes, spleen, or liver after LV infection (data not shown), elevated levels of IL-6 were detected in plasma from

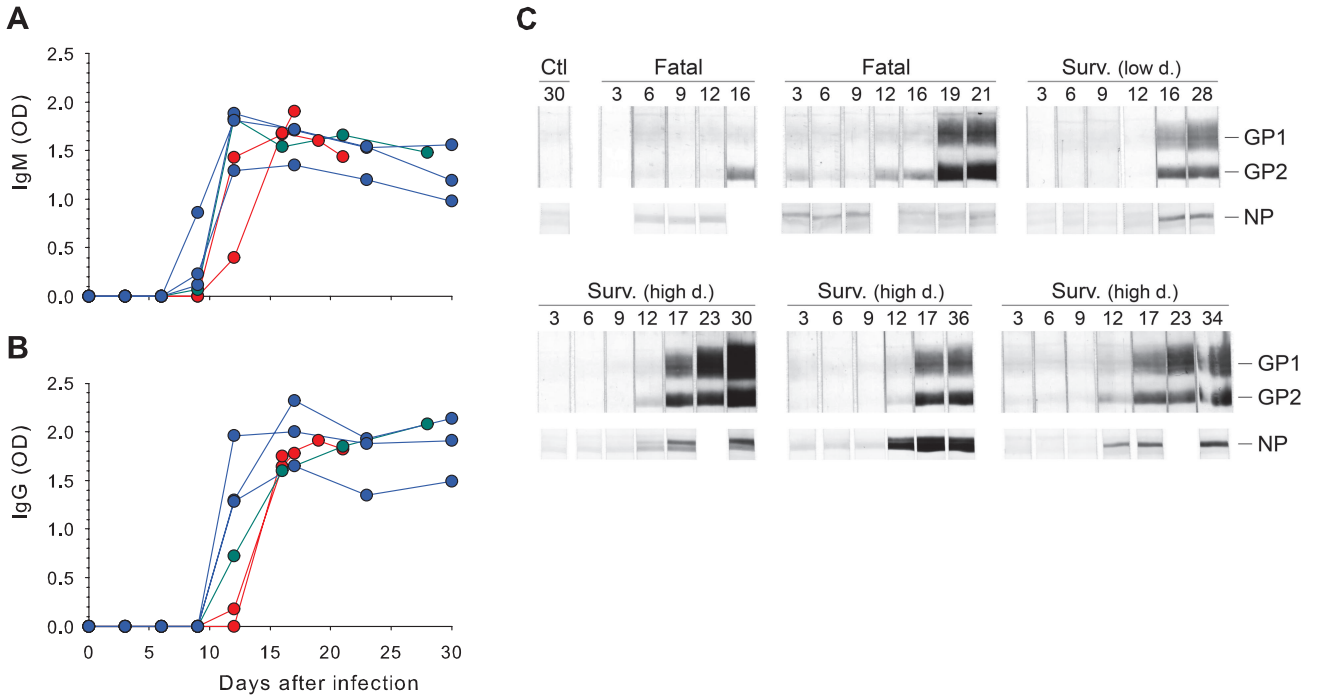

FIG. 4. Analysis of humoral responses during Lassa fever. (A and B) LV-specific IgM (A) and IgG (B) were detected by ELISA in plasma collected during the course of the disease. The results are expressed as optical densities (OD) of plasma at a dilution of 1:1,600 for IgM and of 1:25,600 for IgG. See the legend to Fig. 1 for symbols. (C) Western blotting for NP-, GP_1-, and GP_2-specific IgG in plasma from LV-infected monkeys. The molecular masses of the viral proteins were verified using standard molecular masses and were about 66 kDa for NP, 45 kDa for GP_1, and 38 to 39 kDa for GP_2. The results at various times after LV infection (in days, shown above the lanes) are presented for individual fatally infected monkeys (Fatal) and survivors (Surv.) infected with a low (low d.) or a high (high d.) viral dose. One sample from a mock-infected monkey (Ctl) is shown and is representative of all samples from the two control animals (data not shown).

fatally infected monkeys in the terminal stages, but not in that from survivors (Fig. 6A). Substantial concentrations of IFN-α were found in plasma from animals infected with a high dose of LV as early as 3 days after infection, but only 3 days later in samples from the survivor infected with a low dose and from fatalities (Fig. 6B). Whereas levels of IFN-α dropped rapidly in most survivors, the concentration of IFN-α remained high or increased in the terminal stages in plasma from fatally infected animals. IFN-β mRNA synthesis was induced 3 days after infection in PBMC from two of three monkeys infected with a high dose of LV and was strongly upregulated 3 weeks after infection in PBMC from high-dose-infected animals (Fig. 6C). The amounts of mRNA coding for CCL2, CCL3, CCL4, CCL5, CCL7, and CXCL9 were not modified during LV infection (data not shown). In contrast, high levels of CXCL10 and CXCL11 mRNAs were detected in PBMC from infected monkeys, with the peak 6 days after infection (Fig. 6D and E). Similarly, these mRNAs were also detected at high levels in lymph nodes 9 days after infection (Fig. 6F and G). CXCL10 and CXCL11 mRNAs were more abundant in lymph nodes from fatally infected monkeys than in those from survivors; however, the amounts of these mRNAs in PBMC were similar.

Consequences of the MOI for the replication of LV and for the production of type I IFN. In order to determine the consequences of the viral inocula for viral replication and for the induction of innate immunity, human macrophages were infected at various multiplicities of infection (MOI) with LV, and viral replication and production of type I IFN were studied. Higher viral titers were produced after infection of cells at a low MOI (0.01) than at a high MOI (1 or 10) (Fig. 7A). In addition, infection at a high MOI led to a large increase of the viral RNA/titer ratio (Fig. 7B). Finally, production of IFN-β, IFN-α1, and IFN-α2 was correlated with the MOI (Fig. 7C).

DISCUSSION

Little is known about the pathogenesis of Lassa fever and the associated immune responses. Here, we report some virological, pathological, and immunological variables associated with fatal and nonfatal outcomes after LV infection of cynomolgus monkeys. Both rhesus monkeys (7, 26, 33, 65) and cynomolgus monkeys have frequently been used as models for Lassa fever, and similar clinical signs and pathological events have been observed after LV infection in these closely related species (16, 21, 27, 29, 58). We used two different doses of virus to assess the influence of the viral inoculum on the disease course, and because the AV strain of LV has never been evaluated in primate models. We used the AV strain of LV instead of the more frequently studied Josiah strain because AV is a primary strain recently isolated from a fatal case and had been subjected to only a few passages on Vero E6 cells between isolation from the patient and our experimental animal infections. Thus, this isolate has the advantage of being recently isolated from the field; it is also similar to the prototype Josiah strain, with 82% and 94% homology in the nucleotide and amino acid sequences, respectively (23). The clinical,

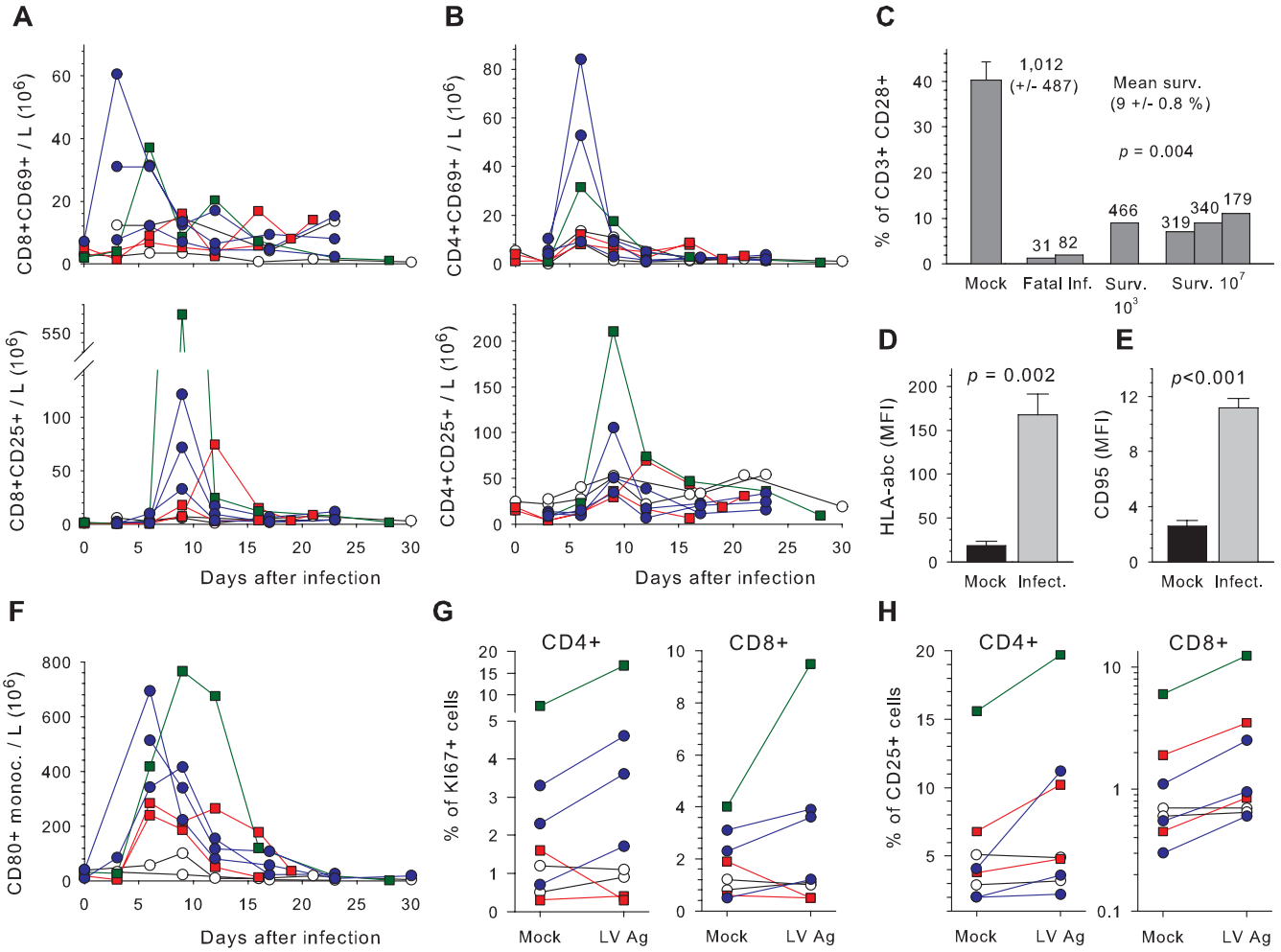

FIG. 5. T-cell and monocyte activation during Lassa fever. The numbers of circulating $CD3^+$ $CD8^+$ cells expressing CD69 (A, top) or CD25 (A, bottom), of $CD3^+$ $CD4^+$ cells expressing CD69 (B, top) or CD25 (B, bottom), and of monocytes expressing CD80 (F) are shown. See the legend to Fig. 1 for symbols. (C) The percentages of $CD3^+$ cells that were $CD28^+$ in the blood from fatally infected monkeys (Fatal inf.) and survivors (Surv.) were determined 16 days after infection. The mean and standard error of all samples ($n = 16$) from mock-infected animals (Mock) is given. The number of $CD3^+$ $CD28^+$ cells is also indicated (number of cells/μl of blood for infected monkeys and mean number/μl ± standard error for control animals). The mean and standard error of data from the four survivors are shown, and significant differences were found with control animals ($P = 0.004$). (D and E) The intensity of expression at the surface of circulating monocytes of HLA-a, -b, and -c (D) and CD95 (E) was evaluated by flow cytometry 6 days after infection in infected monkeys (Infect.) and control animals (Mock). The results are expressed as the mean and standard error of the fluorescence index (MFI) of molecule expression for all infected monkeys ($n = 6$) and for all control samples ($n = 16$). The results of the statistical tests performed to compare control and infected animals are indicated. (G and H) The percentages of lymph node-derived $CD4^+$ and $CD8^+$ T lymphocytes (obtained 9 days after infection of the monkeys) expressing KI67 (G) or CD25 (H) after in vitro mock or inactivated LV stimulation. Note the change of scale in the vertical axis for $CD4^+$ T cells and KI67 and the logarithmic scale for $CD8^+$ T cells and CD25. See the legend to Fig. 1 for symbols.

biological, virological, pathological, and histological findings we observed were similar to those previously described in macaques infected with the Josiah strain of LV, confirming that the AV strain is appropriate for the study of Lassa fever in cynomolgus monkeys. This model allows us to describe several new findings about Lassa fever in macaques. Indeed, whereas previous studies of LV infection in macaques were mainly focused on fatal infection and on pathophysiogenesis, we compared virological and immunological parameters in both fatal and nonfatal outcomes. Here, we described for the first time the relationship between the LV inoculum and the outcome of infection, the number of defective interfering particles (DIP), and the induction of innate immunity. Furthermore, we showed that recovery of LV infection is associated with early and strong innate and adaptive immune responses, illustrated by a high number of activated circulating monocytes and T cells and an induction of LV-specific $CD4^+$ and $CD8^+$ T cells. In contrast, fatal infection is characterized by severe changes in blood composition, weak cellular immune responses, and uncontrolled viral replication.

Our clinical observations are consistent with those previously described in rhesus/cynomolgus monkeys and marmosets infected with the Josiah strain of LV (7, 10, 33): they include fever, anorexia, weight loss, and depression. In addition, acute respiratory syndrome and hypothermia were observed in the terminal stages in moribund monkeys. Although we had to

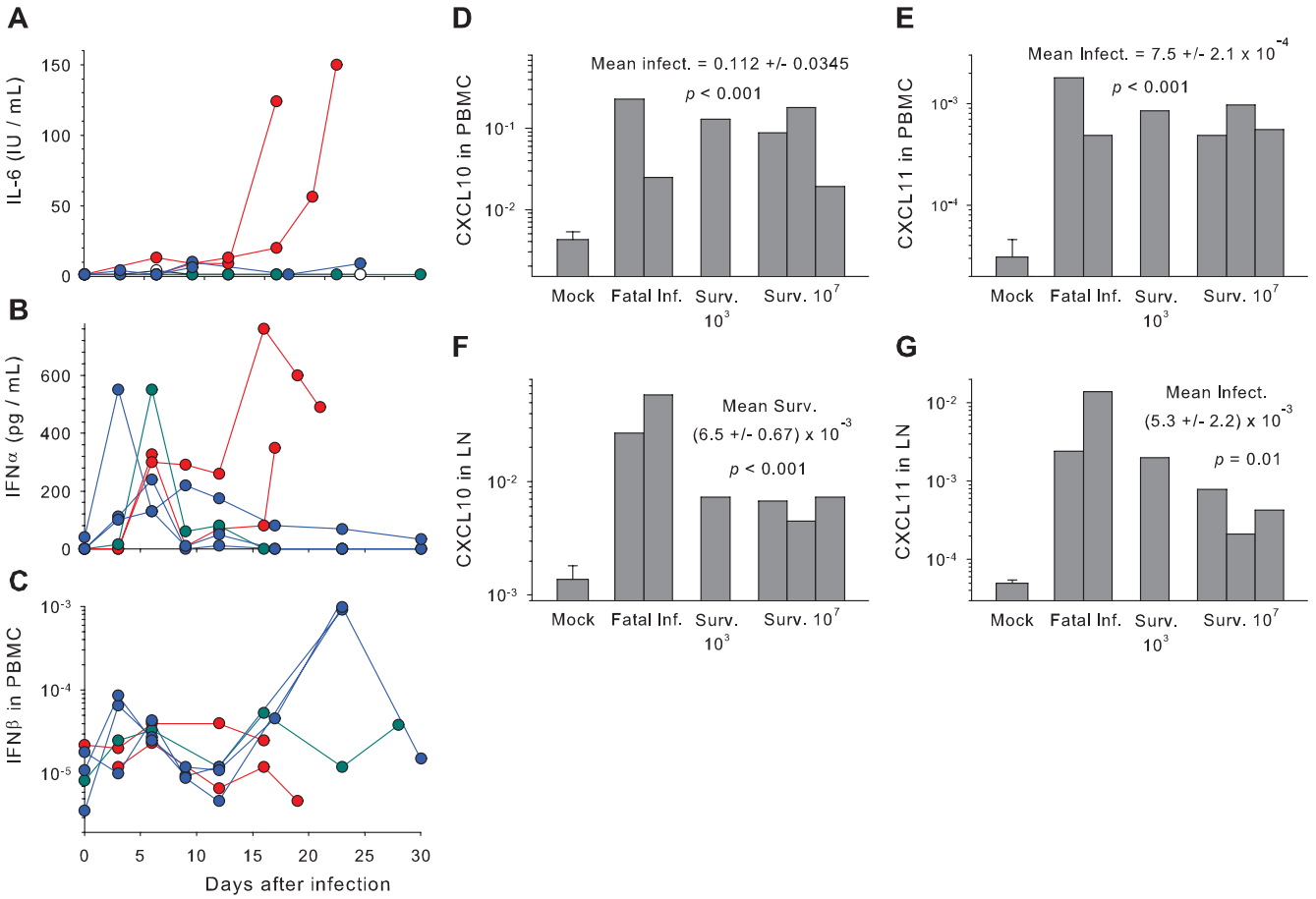

FIG. 6. Detection of the expression of IL-6, type I IFN, and CXC chemokines by ELISA or RT-PCR. (A to C) IL-6 (A) and IFN-α (B) were assayed by ELISA in plasma collected during the course of the disease, and IFN-β mRNA in PBMC obtained during the course of disease was assayed by quantitative RT-PCR (C). The results are expressed as IU/ml (IL-6), pg/ml (IFN-α), or numbers of copies of IFN-β mRNA/number of copies of β-actin mRNA, and the different monkeys are represented as in Fig. 1. IFN-α levels were below the detection threshold of the test in samples from control monkeys, and the relative IFN-β mRNA abundance was $<9 \times 10^{-6}$ for all samples (data not shown). (D and E) The titers of CXCL10 (D) and CXCL11 (E) mRNAs in PBMC obtained 6 days after infection were determined by quantitative RT-PCR (see the legend to Fig. 5C for symbols). The results reported are the numbers of copies of the mRNA considered/number of copies of β-actin mRNA for individual monkeys, except for control animals, for which the mean ± standard error of all samples is given. The mean and standard error of all samples from infected monkeys ($n = 6$) are indicated, as are the results of the statistical test comparing mRNA expression between infected and control monkeys. The titers of CXCL10 (F) and CXCL11 (G) mRNAs were also determined in lymph nodes (LN) obtained 9 days after infection. However, as the expression of CXCL10 was different between fatally infected animals and survivors, the mean for infected monkeys was calculated only with data from surviving animals ($n = 4$).

euthanize one animal for ethical reasons before he succumbed to the disease, we could reasonably assume that this animal would not have recovered from the disease. Indeed, hypothermia, respiratory syndrome, and neurological signs are characteristic of the terminal stages of Lassa fever (7, 65). Furthermore, similar levels of biological markers were detected in this animal and the monkey that succumbed to the disease, with elevated AST/ALT levels, uncontrolled viremia, and high levels of plasmatic IL-6 at the end of the disease. The similarity of the diseases induced by the Josiah and AV strains was not surprising, as the two strains are closely related (23). However, two of the three animals infected with the low viral dose died with Lassa fever, whereas all animals infected with the high dose recovered. The pathological findings and the histological lesions in fatally infected monkeys were consistent with the previous descriptions of the disease induced by the Josiah strain of LV in rhesus/cynomolgus monkeys (7, 65), with lymphadenopathy, splenomegaly, and moderate alterations of the liver, the lungs, and the endothelium. The main microscopic finding was a generalized and substantial infiltration of mononuclear cells, principally macrophages. In contrast, only mild lesions were found in animals with nonfatal infections, suggesting effective control of LV infection. This is consistent with the smaller quantity of viral material found in the organs from survivors than in those from fatalities; note, however, that tissue samples were obtained later after infection from survivors than from fatalities. The titers of replicating LV in most organs from fatalities were high, confirming the pantropism of LV (10, 26, 65, 66). The control of LV replication in survivors was confirmed by the rapid drop in and disappearance of viremia; viral titers in plasma from fatalities rose relentlessly until death. Higher infectious-particle titers were correlated

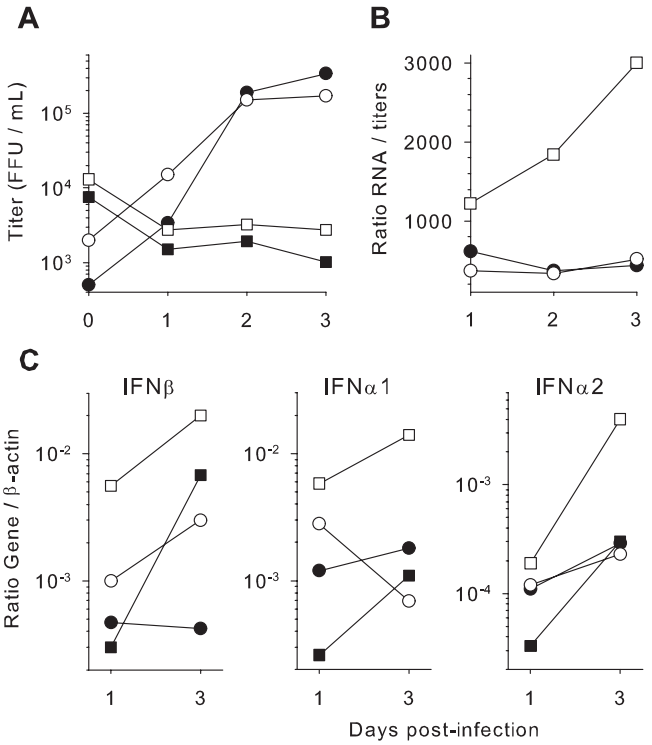

FIG. 7. Consequences of the MOI for the replication of LV and for the production of type I IFN in human macrophages. (A) Macrophages were obtained after differentiation in the presence of macrophage colony-stimulating factor of monocytes purified from healthy human blood, as previously described (2). The macrophages were infected with LV at MOI of 0.01 (black circles), 0.1 (white circles), 1 (black squares), and 10 (white squares), and the titer of viral particles in the supernatants was determined after infection. (B) Viral RNA/titer ratio in culture supernatants. (C) Total cellular RNA was extracted 24 h and 72 h after infection of macrophages with LV at MOI of 0.01 (white circles), 0.1 (black squares), and 2 (white squares) or after mock infection (black circles), and the titers of mRNAs coding for IFN-β, IFN-α1, and IFN-α2 were determined by real-time RT-PCR using primers and probes previously described (3).

with severe disease, as for rhesus monkeys and humans (26, 31). Interestingly, the viral RNA/FFU ratio was significantly higher during the course of the disease in plasma from survivors than in that from fatalities, suggesting that the efficiency of replication was altered in animals that controlled the LV infection. A similar increase in this ratio has been observed in human cells treated with type I IFN (3).

We followed numerous biological markers during LV infection, and only AST and ALT levels were affected. Elevated levels of both enzymes have previously been described during the disease (7, 10, 26, 44, 56); in our study, both fatally infected monkeys and one high-dose survivor showed large increases, whereas the increases were only moderate and transient in the three other survivors. Thus, elevated levels of AST and ALT seem to be associated with death (31, 44) and may be indicative of hepatic abnormalities. However, the elevated AST/ALT ratio suggests that AST was probably produced by organs other than the liver. High levels of IL-6 were detected in the terminal stages in fatally infected monkeys, as previously described in LCMV-infected rhesus monkeys (38, 39). IL-6 production may be linked to hepatic regeneration during severe Lassa fever in monkeys, a phenomenon also described in LV-infected humans (45); thus, the IL-6 titer may serve as a marker of disease severity. Indeed, IL-6 is released after liver injury and is crucial for its regeneration (12). The high IL-6 levels could also result from tissue damage in other organs and muscles, and this would be consistent with the high AST levels observed in fatalities. Interestingly, low but significant concentrations of IL-6 (10 IU/ml) circulated coincidently with the elevated levels of AST/ALT in the survivor in which the concentrations of these enzymes increased substantially. The other hematological and virological markers and the humoral and cytokine responses measured in this animal were comparable to those in the other high-dose-infected monkeys, except that it had the largest number of circulating activated T cells among the high-dose group. We are not able to explain this difference or to link it to the AST/ALT increase in this survivor. It is difficult to reconcile the high levels of IL-6, a pyrogenic cytokine (51), with the absence of significant fever, and indeed the hypothermia, observed in fatalities. However, the most potent pyrogenic cytokine, IL-1β, and also TNF-α, were not detected in these animals. Other mediators, such as anti-inflammatory cytokines or glucocorticoids, may counteract the effect of IL-6 and events downstream from IL-6 signaling, for example, decreased levels of prostaglandin E2, may inhibit fever (49). Transient thrombocytopenia was observed in all infected monkeys, whatever the outcome of the infection, as previously described (17, 33). However, the number of circulating platelets remained low until death in fatalities but returned to baseline values in survivors. The thrombocytopenia could have been caused by either alterations of hematopoiesis or increased splenic pooling, as disseminated intravascular coagulation is not observed during LV infection in primates (33). Failure of the bone marrow to produce platelets is probably the main cause of thrombocytopenia, as suggested by Lange et al. (33). However, one of the main causes of the hemostatic disorders associated with Lassa fever is probably the depression of platelet function, which has been shown to occur coincidently (17, 43). Interestingly, type I IFN has a crucial role in the reduction of platelet counts and in their dysfunction during LCMV infection of mice (24). Unfortunately, we were unable to evaluate platelet functions in our animals. Lassa fever is associated with lymphopenia in humans (17), and leukopenia has been described in nonhuman primates (26, 33). These events are most marked in fatalities and are accompanied by areas of necrosis in lymphoid organs (7, 17, 69). We also observed a transient lymphopenia during the first 2 weeks after infection in all monkeys, and we reported for the first time that both $CD4^+$ and $CD8^+$ T cells, and also B cells and NK cells, were affected. The cause of this lymphopenia is not fully understood, but type I IFN has been implicated in the transient depletion of T and B cells during viral infection, including LCMV infection (30, 32, 47). Similar events probably occur during Lassa fever. The circulating-B-cell count returned to normal levels with similar kinetics whatever the disease outcome, but $CD4^+$ and $CD8^+$ T-cell populations recovered earlier in survivors than in fatalities. Similarly, the drop in the NK cell count seemed more profound and durable in fatally infected monkeys; however, further investigations are required to determine whether NK cells are involved in the control of LV infection. As previously described (17, 18), gran-

ulocytosis, mainly due to neutrophilia (data not shown), was observed in some animals. It has been suggested that CXCL8, a major chemotactic factor for neutrophils (50), is involved in the neutrophilia in LV-infected patients (40), but no overproduction of this chemokine in the plasma or of its mRNA in PBMC or lymph nodes was detected in infected monkeys (data not shown). In the case of fatally infected monkeys, IL-6 may be involved in the neutrophilia: its concentration in plasma was high, and it can induce neutrophilia (54, 63). Interestingly, the increase in the number of circulating granulocytes and in the levels of AST/ALT occurred simultaneously in the fatally infected animals, suggesting that tissue damage may have been at least partially due to infiltrating neutrophils. Consistent with this notion, IL-6 is known to enhance the antibody-dependent cellular cytotoxicity of neutrophils (4).

The control of LV infection does not seem to be related to the induction of NAbs, either in humans or in nonhuman primates (25, 27). Old-world arenaviruses are poor inducers of NAbs, probably because of the intrinsic properties of GP (53). Indeed, we failed to detect NAbs in the animals, even 1 month after infection in survivors, indicating that recovery is not linked to neutralization of LV. Previous studies suggested that the presence of high levels of (nonneutralizing) antibodies was not correlated with disease outcome in humans or rhesus monkeys (16, 26, 31). The presence of high titers of both specific IgM and IgG was confirmed in all animals, but interesting differences were noted according to the disease outcome. First, both LV-specific IgM and IgG appeared earlier and more vigorously in survivors, particularly in the high-dose-infected monkeys, and may therefore help the host to limit and finally control LV replication. It would be interesting to determine whether this phenomenon occurs during human Lassa fever. Second, although high levels of GP_1- and GP_2-specific IgG were detected in all animals, only surviving monkeys produced significant levels of NP-specific IgG, and the most vigorous NP-specific responses were found in the high-dose-infected monkeys. The presence of NP-specific IgM and IgG in plasma from patients has been reported, but it is not known whether there is a correlation between the titer of NP-specific IgG and the disease outcome (62). Thus, the quantitative and qualitative characteristics of the humoral responses of cynomolgus monkeys may contribute to the disease outcome.

A substantial rise in the number of circulating monocytes was observed simultaneously with the decline in virus levels 12 to 16 days after infection in both low- and high-dose survivors, but not in fatally infected monkeys, and was preceded by a large increase in the number of activated circulating monocytes. It is interesting that the most vigorous and lasting monocyte activation occurred in the low-dose survivor. There was a transient increase in the number of circulating NK cells at the same time in the high-dose-infected monkeys. This suggests that activation of monocytes may be important for the control of LV infection. NK cells may also play a protective role, but further investigations would be required to clarify this point, as similar numbers of circulating NK cells were present in the low-dose survivor and fatalities. Macrophages and NK cells may control the replication of LV through production of type I IFN or lysis of infected cells, respectively. LV-infected human macrophages produce fewer viral particles than dendritic cells because of their ability to synthesize small but effective amounts of type I IFN; also, LV release declines after activation of macrophages (3). Furthermore, Mopeia virus, a nonpathogenic arenavirus closely related to LV (65), is able to activate macrophages and induces type I IFN more strongly than LV (52). Production of type I IFN was detected early in monkeys that survived high-dose LV infection, and this could be involved in the rapid control of LV replication. However, a type I IFN response is probably not sufficient to control LV. Indeed, this response was detected at the same time but at higher levels in the low-dose survivor than in fatalities, and high plasma concentrations of IFN-α and high viremia were detected simultaneously 2 weeks after infection in fatally infected animals. Monocytes/macrophages may also help activate LV-specific T cells because of their antigen-presenting-cell properties. Consistent with this, stronger and earlier T-cell responses were observed in survivors than in fatalities, as indicated by the greater numbers of $CD4^+$ and $CD8^+$ T cells expressing CD69 and CD25. Interestingly, the highest number of $CD25^+$ T cells was detected in the low-dose survivor. Like the humoral response, T-cell activation was detected about 3 days later in fatally infected animals and at lower levels. The presence of LV-specific $CD4^+$ and $CD8^+$ T cells in survivors, and to a lesser extent in fatalities, was confirmed after in vitro stimulation of lymph node-derived lymphocytes with LV Ag. Indeed, cell proliferation was observed only with T cells from survivors, but not those from fatally infected animals, whereas CD25 expression was detected in T cells from all infected monkeys. The best responder to antigenic stimulation was the low-dose survivor. This is consistent with the strong T-cell activation observed ex vivo in its PBMC and suggests that a robust T-cell response is associated with survival after low-dose LV infection. These various observations suggest that, in addition to a T helper response probably involved in the induction of a humoral response, a cytotoxic-T-cell response was induced in survivors a few days before the drop in viremia. These responses are probably important for the control of LV replication and recovery. This is consistent with the presence of $CD4^+$ T cells specific for NP and GP in LV-seropositive subjects living in zones of endemicity (60, 61). Interestingly, a strong and significant drop in the number of T lymphocytes expressing CD28 was observed 16 days after infection in the blood of infected monkeys. This may reflect the differentiation of T lymphocytes into memory cells (55). However, the disappearance of CD28 expression was more pronounced in fatalities and affected most circulating T cells. Thus, it may be related to the reconstitution of the lymphocyte pool after lymphopenia, which is known to induce a "memory-like" phenotype in homeostasis-driven proliferating T cells (22, 64).

CXCL10 and CXCL11 mRNAs were abundant in PBMC and lymph nodes from infected monkeys. These chemokines are mainly produced in response to type I and type II IFN and are able to attract activated T cells to inflamed sites (48). In our animals, the intense mononuclear cell infiltrates suggest that these chemokines were probably mainly produced by monocytes/macrophages, but endothelial or epithelial cells may also participate. Although elevated levels of CXCL10 have been correlated with nonfatal outcomes of human Lassa fever (40), we observed similar expression of CXCL10 mRNA, and even higher in lymph nodes, in fatally infected animals and survivors. This issue therefore remains unclear, but the stron-

ger expression of mRNA in lymph nodes in fatalities may be due to the intense viral replication that occurred in these organs. Despite the massive macrophage infiltrate in fatalities and the activation of immune cells in survivors, we failed to detect macrophage- or T-cell-derived cytokine mRNAs or proteins. This is difficult to explain, but cytokines could have been produced only locally at low levels in infected tissues. Unfortunately, in situ detection of cytokines in organs and tissues was not performed. However, most of the macrophages were probably infected in the terminal stages, which may explain the lack of inflammatory cytokine synthesis. Indeed, LV infection of macrophages and dendritic cells is not associated with the release of inflammatory cytokines (2), and Lassa fever in humans seems not to be associated with massive inflammatory and T-cell-derived cytokine release (40).

The unexpectedly more severe disease associated with the low viral dose is difficult to explain. No clear association between the LV inoculum and disease outcome has been observed previously in rhesus monkeys (7, 18, 26, 27, 33, 65). These different outcomes may be explained by the simultaneous production of both infectious particles and DIP during LV replication. Indeed, a large number of DIP are released during the replication of negative-strand RNA viruses (34), and this phenomenon has been extensively described for LCMV and other arenaviruses (14, 67). A large number of DIP seem also to be present in LV stocks, as suggested by the high viral RNA/FFU ratio (about 100 to 200) (our results and reference 1) observed. DIP inhibit the production of infectious particles, and this interference is stronger at high MOI (5). Similar inhibition has been observed with LV, as higher viral titers were obtained after infection of Vero E6 cells (unpublished results) and of human macrophages at a low MOI than at a high MOI. Furthermore, infection of macrophages at a high MOI led to a large increase in the viral RNA/FFU ratio and, thus, the DIP/infectious-particle ratio. Administration of the high dose to monkeys probably led to significant interference in LV replication, and this is consistent with the higher viral RNA/FFU ratio observed in these animals than in the fatalities. In addition, DIP have been reported to be associated with stronger induction of innate responses (20, 42). Thus, infection with a high dose, but also the consequently higher DIP/infectious-particle ratio, probably led to a stronger induction of innate than adaptive immunity. This could help animals to limit viral dissemination rapidly and thereby avoid severe symptoms. The detection of type I IFN production earlier in the high-viral-dose-infected monkeys than in fatalities and in the low-dose-infected survivor is also consistent with this suggestion. In addition, synthesis of type I IFN mRNA by LV-infected human macrophages was correlated with the MOI. Type I IFN may also be involved in the increase in the viral RNA/FFU ratio observed in survivors, as similar alterations of the efficiency of LV replication have been described after stimulation of human cells with type I IFN (3). Considering the mode of transmission of LV in humans, it is unlikely that individuals would be infected with large doses of virus, and thus, the data obtained in monkeys infected with the large dose have only limited relevance to the situation observed in the field. Nevertheless, the high-dose-infected monkeys not only help us to understand virus-host interactions, but also constitute a model of nonfatal Lassa fever and associated protective immune responses that are induced against LV.

In conclusion, fatal infections were associated with large alterations to the blood formula and organs, delayed humoral responses, and weak T-cell and monocyte activation, leading to elevated and uncontrolled viral replication. In contrast, nonfatal infections were characterized by early and strong innate, humoral, and T-cell responses, vigorous activation of monocytes, and effective control of viral replication. Finally, administration of a high viral dose seemed to favor a nonfatal issue of Lassa fever in cynomolgus monkeys, and this may have been due to the presence of DIP.

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